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Isolation and Characterization of Wheat Straw Lignin

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A method was developed for the isolation of lignin from wheat straw by ball milling and enzyme treatment. Grinding duration, cellulase hydrolysis time, and dioxane–water composition of the extraction solvent on lignin yield were examined. Ball-milling for 8 days followed by cellulose hydrolysis for 4 days were needed to maximize isolated lignin yield. Extraction of ball-milled, enzyme-treated straw with 50% dioxane–water resulted in twice as much lignin solubilization as seen for a 96% dioxane–water extraction. Nitrobenzene oxidation, solid-state and solution NMR, and infrared spectroscopy of isolated lignins indicated few differences between lignins in the various fractions. Lignin soluble in 96% dioxane had the least carbohydrate contamination and the highest concentration of cinnamic acids. Progressively more carbohydrate and less cinnamic acids were found in the 50% dioxane-soluble and water-soluble lignin fractions. Some acetyl groups and ethanol were found in the lignins. Lignin yields were high from this isolation procedure, and the data suggest that 50% dioxane lignins from herbaceous plants result in greater yields than 96% dioxane without major changes in lignin composition.

Although lignin has long been associated with poor forage fiber digestion by ruminant animals, the mechanism(s) of this inhibition has never been established (Jung and Fahey, 1983). Evidence is accumulating that chemical composition and structure of forage lignin plays a larger role in determining fiber digestibility than simply quantity of lignin. The relationship between forage digestibility and lignification is consistently different between legumes and grasses (Mowat et al., 1969). It was found that legume stems were much more digestible than grass stems of equal lignin content, and legumes contain lignin which has smaller amounts of sinapyl alcohol units (Jung et al., 1983b). As grasses mature, the inhibitory effects of additional lignin concentration become progressively smaller (Van Soest, 1967; Jung and Vogel, 1986) and a similar affect is seen for legumes, but the decline in lignin's effect on digestibility is sharper (Jung, H. G., 1986). It has been

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shown that as tall fescue matured, extractability of nitrobenzene oxidation products of lignin increased (Jung et al., 1983b). The brown midrib mutants of corn and sorghum usually contain less lignin than the normal genotype and are more digestible by ruminants (Muller et al., 1972; Porter et al., 1978). However, even when lignin concentrations were the same between the corn genotypes, the brown midrib mutant was still more digestible by cattle (Sommerfeldt et al., 1979). Indications are that etherated syringyl content is greatly reduced in the brown midrib mutant of sorghum (Akin et al., 1986). Analysis of brown midrib corn has shown that the extractability of aromatic aldehydes is less, especially syringaldehyde (Gee et al., 1968; Cymbaluk et al., 1973; Grand et al., 1985). Unfortunately, available data on chemical structure of forage lignins are very poor and do not allow definitive interpretation of these observations.

The chemical structure of wood lignin is much better known, and a wealth of techniques are available for characterization of wood lignin (Sarkanen and Ludwig, 1971). The objective of this study was to develop a method for isolation of relatively unmodified lignin from forage to use in more extensive chemical characterization work, similar to what has been accomplished with wood (Chang et al., 1975).

MATERIALS AND METHODS

Wheat straw (*Triticum aestivium*) was chosen as the forage source for development of the lignin isolation procedure as it is high in lignin and is generally poorly digested. The straw was ground through a 1-mm screen prior to analysis. Total cell wall fiber (neutral detergent fiber), hemicellulose (neutral detergent fiber minus acid detergent fiber), cellulose (acid detergent fiber minus 72% H_2SO_4 acid detergent lignin), and lignin (acid detergent lignin minus ash) were determined by the sequential detergent procedure (Van Soest and Robertson, 1980). Crude protein was measured as Kjeldahl N × 6.25 (AOAC, 1975). Neutral detergent fiber (Van Soest and Robertson, 1980) was prepared from the wheat straw for subsequent analysis. Organic matter was determined by ashing at 500 °C for 3 h.

Grinding Time. A 50-g portion of wheat straw neutral detergent fiber was ground in an Attritor agitating ball-mill (Union Process, Akron, OH). The 750-mL pot was filled with the sample and 1700 g of 3.06-mm stainless steel balls. Sufficient toluene was added to cover the sample and grinding media. The sample chamber was cooled by a water jacket. Grinding speed was set at half full range (~300 rpm). Samples were taken after 1, 2, 4, 6, 8, 12, and 16 days of grinding. The entire contents of the sample chamber were removed at each sampling time. The sample was strained through a 1-mm screen to remove the stainless steel balls, and then a 25-mL aliquot of the sample slurry was removed for analysis. The rest of the sample was returned to the sample chamber for continued grinding. The 25-mL samples were filtered through Whatman 54 filter paper (Whatman Paper Ltd.). The ball-milled residue was then allowed to air-dry prior to lignin extraction.

Cellulase Hydrolysis Time. The ball-milled residue of wheat straw ground for 8 days was used to determine optimum hydrolysis time with cellulase. The cellulase was derived from *Aspergillus niger* (0.75 mmol of glucose/h from cellulose; Type II; Sigma, St. Louis, MO) and is reported to contain substantial hemicellulose activity. The sample was suspended in 20 mL of 33 mM acetate buffer, pH 4.5, per gram sample. The acetate buffer contained 5 $mg \cdot mL^{-1}$ cellulase for a substrate to enzyme ratio of 10:1. This ratio of substrate to enzyme was a compromise between activity and amount of enzyme needed for large batchs of substrate. A few drops of toluene were added as a preservative. Samples were incubated at 39 °C for 1, 2, 4, 6, or 8 days. Samples were filtered, washed with water, and air-dried prior to lignin extraction of the hydrolysis residue.

Lignin Extraction. Wheat straw samples from the grinding time and hydrolysis time experiments were extracted with 96% (v/v) dioxane–water. The samples were extracted with 10 $mL \cdot g^{-1}$ sample weight at room temperature for 24 h. After filtration, the extracts were diluted 1:100 with 96% dioxane and absorbance at 280 nm was measured.

The influence of solvent composition on lignin extraction was determined by extracting samples ball-milled for 8 days only or ball-milled and hydrolyzed with cellulase for 4 days. Samples were extracted as before with dioxane–water mixtures containing 0, 20, 40, 60, 80, 96, or 100% dioxane. Efficiency of lignin extraction was measured as absorbance at 280 nm.

Lignin Isolation. Wheat straw lignins were isolated for chemical characterization by ball-milling the straw for 8 days, followed by hydrolysis with cellulase for 4 days, and successive extraction of the residue with 96 and 50% dioxane. The extraction solvent was removed from the 96 and 50% extracts by lyophilizing. The residues were then redissolved in a minimal amount of 90% acetic acid. The 96 and 50% dioxane-soluble, water-insoluble lignins were precipitated by adding the acetic acid solutions to large volumes of water. The lignins were recovered by centrifugation and lyophilized. The aqueous supernatants from the lignin precipitation steps were also lyophilized to recover the dioxane-soluble, water-soluble lignin fractions.

Lignin Characterization. Composition of the isolated wheat straw lignins was determined by nitrobenzene oxidation (Jung et al., 1983a). Duplicate 10-mg lignin samples were treated with 1 mL of nitrobenzene and 10 mL of 2 N NaOH at 160 °C for 3 h. Samples were then filtered and washed with ether and water, and the filtrates were combined. Nitrobenzene and its degradation products were removed by ether extraction of the sample filtrate. The phenolic products of oxidation were recovered by acidification of the aqueous sample and extraction with ether. Phenolic compounds were measured by liquid chromatography as described by Jung et al. (1983a).

Solid-State NMR Spectroscopy. Solid-state NMR spectra were obtained with use of the CP/MAS technique. A JEOL FX-270 NMR spectrometer operating at 67.8 MHz for ^{13}C equipped with high-power amplifiers and a narrow-bore probe supplied by Chemagnetics was employed for this purpose. Only spectra of the dioxane-soluble lignins were obtained.

The samples were packed in 7-mm Kel-F rotor and spun at 2.5 kHz. Data were collected by taking 16 384 scans using a 7.0-μs 90° ^1H pulse, a 2-ms contact time, and a 2-s delay time. The total sideband suppression (TOSS) technique (Dixon, 1982) was employed to suppress ^{13}C sidebands encountered under these instrumental conditions. The 2K of data points (zero-filled to 8K) were collected with a 16-kHz spectral window. The data were processed with 30-Hz line broadening to improve S/N. The spectra were referenced to poly(dimethylsilane) (Petrarch Systems, Inc.) at −1.84 ppm with respect to Me_4Si. Of the reference, 5 mg was placed (wrapped in Teflon tape) at one end of the rotor.

Solution NMR Spectroscopy. Solution NMR spectra were obtained on a Bruker AM500 spectrometer operating at 125.7 MHz for ^{13}C. All spectra were obtained under

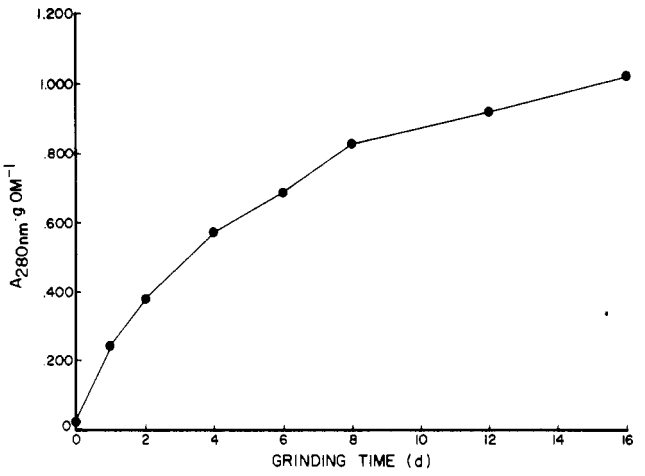

Figure 1. Effect of length of grinding in ball-mill on extractability of lignin with 96% (v/v) dioxane–water.

conditions of complete decoupling, accumulating 26 312 scans into 16K data points. A 4-Hz line broadening was used to improve S/N. Dioxane-soluble, water-insoluble samples were dissolved (50 mgs/0.5 mL) in ^{12}C-enriched hexadeuteriodimethyl sulfoxide ([^{12}]Me_2SO-d_6; Merk, Sharp and Dohme) with about 1% Me_4Si added as an internal reference at 0 ppm. Water-soluble samples were dissolved (50 mg/0.4 mL) in 50% [^{12}C]Me_2SO-d_6–water mixture, with the center band of [^{12}C]Me_2SO-d_6 being used as the internal reference at 39.6 ppm. A distortionless enhancement by polarization transfer (DEPT) sequence (Doddrell et al., 1982) was utilized to confirm the assignments of CH_3, CH_2, and CH carbons in the spectrum of 96% dioxane lignin.

Infrared Spectroscopy. Infrared (IR) spectra were obtained on an Analect fx-6160 FT-IR spectrometer. Samples were run in the single-beam transmission mode as KBr disks (2 mg of sample/58 mg of KBr). Each spectrum was the result of 128 scans subtracted from a background of 128 scans of IR-grade KBr under ambient conditions. Spectral data were accumulated at 4-cm^{-1} resolution over the range 4012–401 cm^{-1}. Data were processed with trapezoidal apodization and then displayed in the absorbance mode with base-line correction and 15-point smoothing over the range 3100–600 cm^{-1}.

RESULTS AND DISCUSSION

The wheat straw contained 77.2% cell wall material and 5.7% crude protein. The composition of the cell walls was 36.7% hemicellulose, 52.8% cellulose, and 8.0% lignin. Extractability of lignin and other cell wall phenolics with 96% dioxane was found to still be increasing even after 16 days of ball-milling (based on absorbance at 280 nm) (Figure 1). When a quadratic model was fit to these data, it was found that 22–24 days would be required to reach maximum lignin extractability. Sarkanen and Ludwig (1971) reported that 40–50 days of milling is required for maximum lignin extraction from conifer wood using a roller-type ball-mill. Vibratory ball-mills are reportedly more efficient in reducing particle size and degree of polymerization (Björkman, 1956). Grinding times of 40–50 h were used with a vibratory ball-mill by Chang et al. (1975) to extract wood lignins. Eight days was chosen as the grinding time for further work in the current study as it was a convenient length of time and allowed extraction of 70+% of the predicted maximum extractable lignin after extended ball-milling.

Chang et al. (1975) used 6-day cellulase hydrolysis to improve yield of sweetgum and spruce lignins by 248 and 165%, respectively. Figure 2 illustrates the effect of cellulase hydrolysis on extraction of wheat straw lignin and associated cell wall phenolics. The majority of the increase in yield (240%) is seen after only 1 day of treatment. A cellulase treatment time of 4 days was chosen in this study for subsequent work because 94% of the maximum observed improvement was obtained after this time.

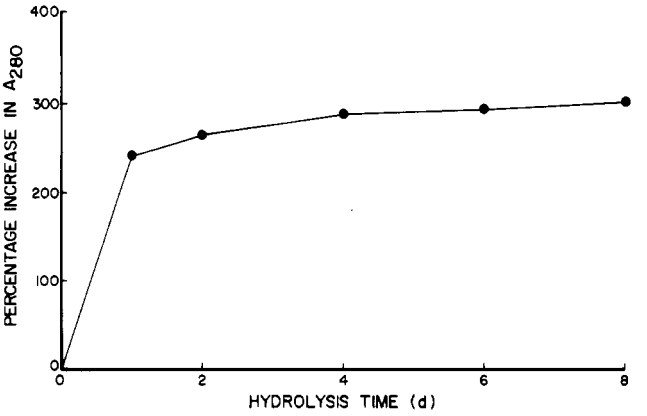

Figure 2. Increase in extractability of lignin with 96% (v/v) dioxane–water after hydrolysis with crude cellulase for various lengths of time.

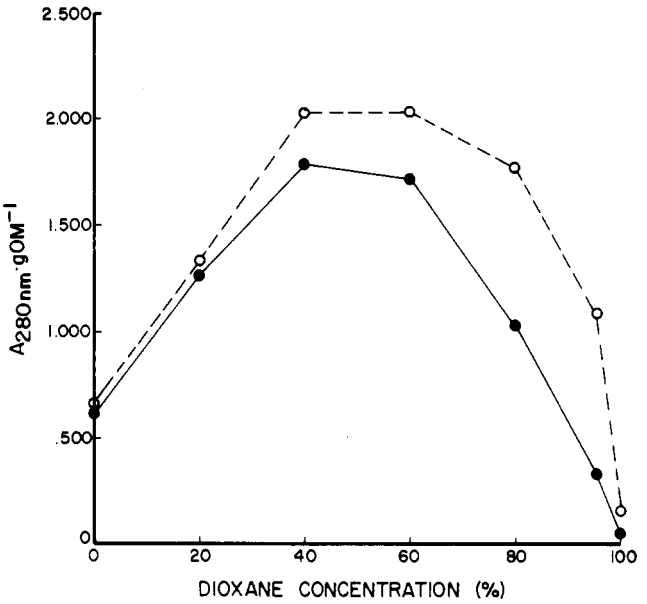

Figure 3. Effect of dioxane concentration of solvent on extraction of lignin from wheat straw: (- - -) ball-milled and hydrolyzed; (—) ball-milled only.

Although 96% dioxane is the standard solvent for lignin extraction from wood, Chang et al. (1975) found that 50% dioxane gave higher yields as measured by 280-nm UV absorbance. A similar result was found for wheat straw lignin (Figure 3). The difference in lignin extractability between samples that were only ball-milled and those that were ball-milled and hydrolyzed with cellulase was greatest for solvent strengths from 80 to 96% dioxane. Pure water actually extracted more 280-nm UV-absorbing material than 96% dioxane from ball-milled wheat straw, but presumably this material was low molecular weight phenolic–carbohydrate complexes. Since absorbance at 280 nm is not specific for lignin, the other cell wall phenolics will influence our results.

During the isolation of wheat straw lignin for characterization, it was found that 12.0% of the organic matter was lost during the 8-day ball-milling step. The subsequent 4-day cellulase hydrolysis and sequential extraction with 96 and 50% dioxane removed an additional 75.3%

of the organic matter. The amount of lignin isolated from 40 g of ball-milled wheat straw was 1.67 g of 96% dioxane-soluble, water-insoluble lignin and 1.45 g of lignin soluble in a subsequent 50% dioxane extraction. In addition, 300 mg of lignified material was recovered from the residual aqueous phase of the purification step of the 96% dioxane soluble material, and 100 mg of lignified material was recovered from the aqueous phase of the 50% dioxane extraction. These lignin preparations were all found to be ash-free.

Accurate calculation of total lignin recovery as a percentage of that present in the original wheat straw proved impossible because we were unable to obtain repeatable measure of acid detergent lignin content of the ball-milled wheat straw. As a result, it is not known whether the loss of 12% of the organic matter during ball-milling enriches or depletes the lignin content of the subsequent material. If it is assumed that lignin is lost during ball-milling in proportion to its content in total cell wall organic matter, then lignin recovery through the isolation procedure was 126.1%. Obviously this value is an overestimate, and it is assumed that the portion of the total organic matter not solubilized in the isolation procedure (13%) contains some lignin. The major contaminate of these lignin preparations was undoubtably carbohydrates, in the form of lignin–carbohydrate complexes. Chang et al. (1975) found 3–9% carbohydrate in wood lignin preparations. The spectral data discussed later confirm this assumption. There may also be some nitrogenous compounds contaminating the lignin as the wheat straw cell walls (NDF) contained 0.46% nitrogen, but the nitrogen concentration of the insoluble residual material was only increased to 0.61%. Further work is required to quantify the true recovery of lignin by this procedure.

The simple phenolic monomers recovered after nitrobenzene oxidation of the wheat straw lignin preparations are given in Table I. The two benzoic acids (*p*-hydroxybenzoic and vanillic acids) accounted for 1% or less of the lignin in all the preparations. In contrast, the cinnamic acid derivatives accounted for 0.01–12% of the total lignin, with the water-soluble 50% dioxane lignin yielding the least cinnamic acids and the water-soluble 96% dioxane containing the greatest amount. The 96 and 50% dioxane-soluble, water-insoluble lignins were intermediate. Cinnamic acid concentrations were low, and repeatability of analysis was poor. The 96 and 50% dioxane-soluble, water-insoluble lignins had similar *p*-coumaric to ferulic acid ratios (0.95 and 0.87, respectively), whereas the water-soluble 96% dioxane lignin had a much lower ratio (0.26) and the water-soluble 50% dioxane lignin contained no ferulic acid. Recovery of the aromatic aldehydes, which are the major products of nitrobenzene oxidation of wood, was low and variable (9–14% of the lignin). The syringyl to coniferyl unit ratio for aldehydes declined from 96% dioxane-soluble, water-insoluble lignin to the 50% dioxane soluble, water-soluble lignin (Table I). The water-soluble, 96% dioxane lignin contained the greatest amount of *p*-hydroxybenzaldehyde. Total yield of nitrobenzene oxidation products was greatest for water-soluble 96% dioxane lignin and least for the water-insoluble 50% dioxane preparations.

The structure and composition of samples of the two dioxane-soluble, water-insoluble lignins were first investigated spectroscopically by solid-state NMR, since it is a nondestructive method of analysis. The solid-state NMR spectra indicated that these two fractions contained primarily lignin. This was indicated by signals in the regions 50–60, 70–90, 95–130, 130–140, and 140–160 ppm in both

Table I. Concentrations of Nitrobenzene Oxidation Products in Lignin Preparations Isolated from Wheat Straw (Grams per Kilogram of Lignin)[a]

	benzoic acids			cinnamic acids				benzaldehydes			
sample	PHBA	VA	VA:PHBA	CA	PCA	FA	PCA:FA	PHBAL	VAN	SYAL	SC ratio[b]
96% dioxane	1.19 ± 0.46	9.48 ± 2.14	7.97	11.3 ± 10.57	39.47 ± 1.87	41.48 ± 2.47	0.95	3.65 ± 0.73	50.66 ± 4.34	54.26 ± 5.75	1.07
50% dioxane	0.80 ± 0.12	8.21 ± 0.25	10.25	0.17 ± 0.17	28.89 ± 0.26	33.12 ± 1.49	0.87	2.91 ± 0.06	44.20 ± 6.61	44.48 ± 4.08	1.01
water-soluble 96% dioxane	0.99 ± 0.25	9.18 ± 1.08	9.27	0.11 ± 0.11	23.54 ± 0.83	91.60 ± 3.01	0.26	14.94 ± 0.40	67.55 ± 2.21	62.80 ± 1.30	0.93
water-soluble 50% dioxane	0.75 ± 0.32	3.80 ± 2.03	5.07	0	1.06 ± 0.02	0		1.90 ± 0.59	70.10 ± 0.94	56.10 ± 3.67	0.80

[a] Key: *p*-hydroxybenzoic acid, PHBA; vanillic acid, VA; cinnamic acid, CA; *p*-coumaric acid, PCA; ferulic acid, FA; *p*-hydroxybenzaldehyde, PHBAL; vanillin, VAN; syringaldehyde, SYAL. [b] Ratio of syringyl to coniferyl units.

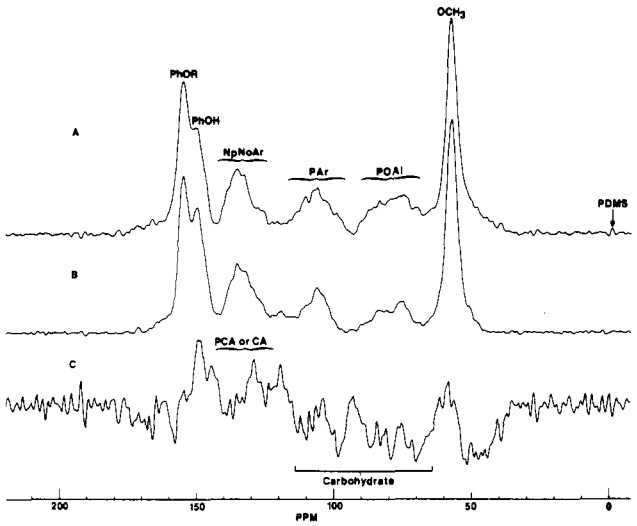

Figure 4. Solid-state ^{13}C NMR spectra of dioxane lignins: (A) 50% (v/v) dioxane–water; (B) 96% (v/v) dioxane–water; (C) difference 96% minus 50% dioxane lignins. Key: PDMS = poly(dimethylsilane), OCH_3 = methoxyl, POAl = protonated oxygenated aliphatic, PAr = protonated aromatic, NpNoAr = nonprotonated nonoxygenated aromatic, PhOH = phenolic (nonetherfied), PhOR = phenolic (etherified).

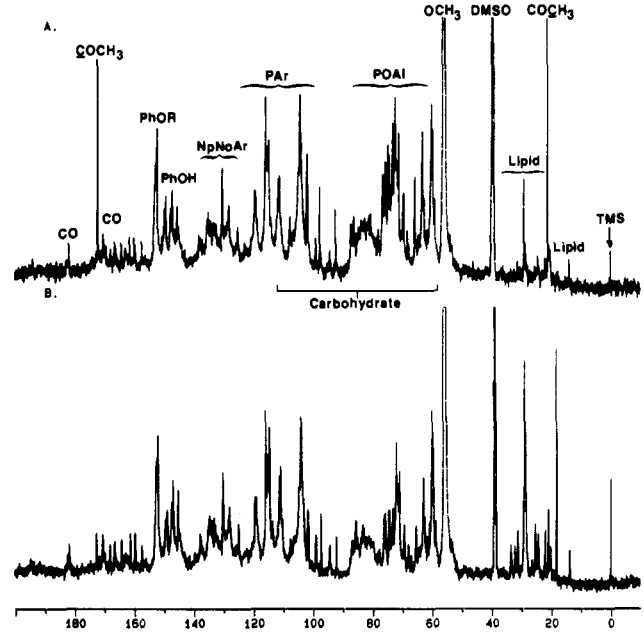

Figure 5. Solution ^{13}C NMR spectra of dioxane lignins: (A) 50% (v/v) dioxane–water; (B) 96% (v/v) dioxane–water. Key: TMS = tetramethylsilane, $COCH_3$ = methyl in acetyl, DMSO = dimethyl sulfoxide, OCH_3 = methoxyl, POAl = protonated oxygenated aliphatic, PAr = protonated aromatic, NpNoAr = nonprotonated nonoxygenated aromatic, PhOH = phenolic (nonetherfied), PhOR = phenolic (etherified), CO = carbonyl, $COCH_3$ = carbonyl in acetyl.

spectra (Figure 4A,B). These are primarily due to aromatic methoxyl, protonated aliphatic, protonated aromatic, nonprotonated nonoxygenated aromatic, and nonprotonated oxygenated aromatic carbons, respectively. These signals are consistent with signals previously observed in solid-state NMR spectra of lignin (Maciel et al., 1981; Himmelsbach et al., 1983; Hatfield et al., 1987; Leary et al., 1986). Carbohydrates may be contributing to signals in the 70–90 and 95–110 ppm regions. The finding by Chang et al. (1975) that wood lignins isolated by these procedures still contain small amounts of carbohydrates (3–9%) is consistent with a small contribution from carbohydrates in these spectra. Six signals that may be related to carbohydrate (at 70, 75, 86, 99, 108, and 110 ppm) increase in intensity in the 50% dioxane lignin spectrum over that of 96% dioxane lignin. This is shown by negative signals in the difference spectrum (Figure 4C) obtained by a 1:1 computer subtraction. The difference spectrum also indicates that there is a difference in the aromatic nature of the two preparations. The positive signals at 119, 129, and 145 ppm suggest there are fewer *p*-coumaryl (PCA) or cinnamic acid (CA) units in the 50% dioxane preparation relative to the other phenolic type units. It should also be noted that there is a very high ratio of etherifed units to nonetherifed phenolic units in both preparations, as indicated by the intensity ratio of signals centered at 154 and 148 ppm, respectively.

Since there were insufficient amounts of the dioxane-soluble, water-soluble samples for solid-state NMR, all samples were then subjected to the more intrusive solution-state NMR spectroscopy. In order to keep all samples in essentially the same solvent system, $[^{12}C]Me_2SO$-d_6 was chosen as the solvent. The spectra of the dioxane-soluble, water-insoluble lignin preparations are shown in Figure 5. Most of the observed signals have been previously assigned in other lignin spectra (Himmelsbach and Barton, 1980). These spectra also generally reflect the same signals observed in the solid-state, except that they are more resolved and have somewhat different relative intensities, a typical result due to the different mechanisms by which the magnetization is generalized for the two techniques. The signals are also shifted by about −2 ppm, in general, from those in the solid state. Here there are additional signals dectected for lipid or waxes (at 14 and 22–35 ppm; Barton et al., 1975, 1978), ethanol (at 18 and 56 ppm), and acetic acid or acetate (at 21 and 173 ppm) that were not visible by solid-state NMR. Also there is a signal for the solvent (at 39 ppm) that was, of course, not present before. The spectrum of the 96% dioxane-soluble lignin (Figure 5B) shows that this preparation contains more lipid or wax than the 50% dioxane lignin and that there is ethanol present, probably resulting from the enzymatic digestion of the carbohydrates. It also shows slightly less carbohydrate. On the other hand, the spectrum of the 50% dioxane lignin preparation shows that it is contaminated with residual acetic acid or is partially acetylated. The other differences observed are the same as in the solid state.

The spectra of the dioxane-soluble fractions that were also water soluble are shown in Figure 6. The spectrum of the water-soluble lignin fraction from the 50% dioxane preparation (Figure 6A) shows that it contains a large amount of carbohydrate, relative to the other samples, but that the lignin structure has remained essentially the same as the parent dioxane-soluble fraction (except again for the loss of PCA or CA). The spectrum of the water-soluble fraction from the 96% dioxane preparation (Figure 6B) shows that it has essentially the same lignin structure (except it, like the parent 50% dioxane preparation, shows a loss of PCA and CA) but contains more carbohydrate, less lipid or waxes, and very little ethanol relative to the dioxane-soluble fraction. Spectrally the structure of lignin thus appears to remain the same in dioxane lignin preparations, except for the apparent loss of phenolic acids. According to the NMR results, the loss of PCA and CA is in the order 96% dioxane lignin < 50% dioxane lignin < 96% water-soluble lignin < 50% water-soluble lignin. This result is supported by the chemical data (Table I). This appears to be the only spectroscopically observable change in lignin structure among all of the preparations.

This brings us back to the old question of whether or not phenolic acids are actually part of the polymeric lignin.

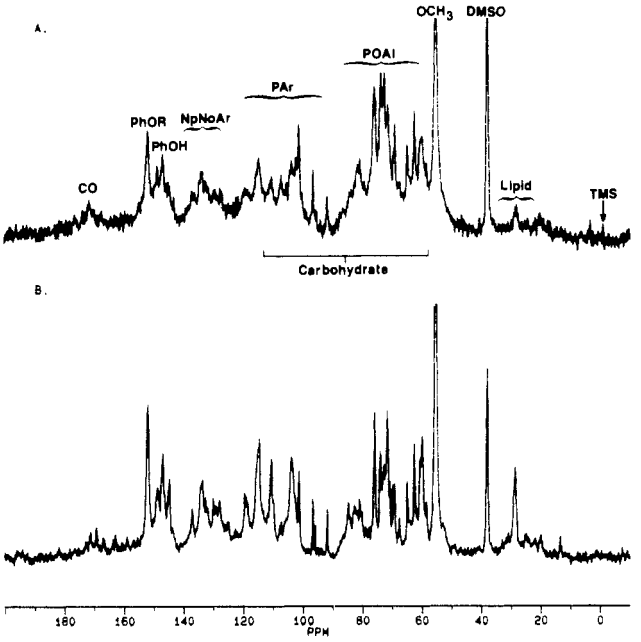

Figure 6. Solution ^{13}C NMR spectra of water-soluble fractions from dioxane lignins: (A) 50% (v/v) dioxane–water; (B) 96% (v/v) dioxane–water. Key: TMS = tetramethylsilane, DMSO = dimethyl sulfoxide, OCH_3 = methoxyl, POAl = protonated oxygenated aliphatic, PAr = protonated aromatic, NpNoAr = nonprotonated nonoxygenated aromatic, PhOH = phenolic (nonetherfied), PhOR = phenolic (etherified), CO = carbonyl.

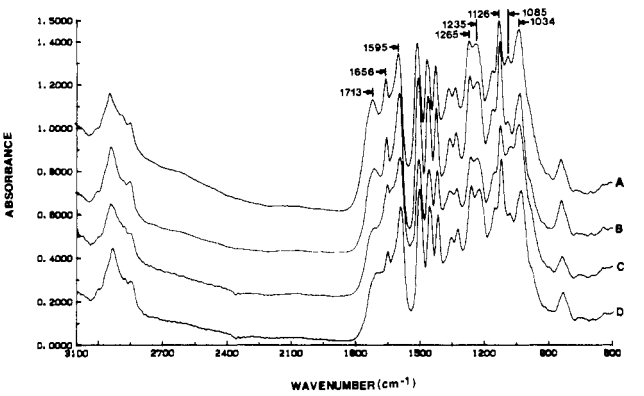

Figure 7. Fourier transform infrared spectra of lignin preparations: (A) 50% (v/v) dioxane–water; (B) 96% (v/v) dioxane–water; (C) water-soluble fraction from 50% (v/v) dioxane–water; (D) water-soluble fraction from 96% dioxane–water. Each spectrum is offset from the spectrum below it by 0.2 absorbance unit.

If they are, then the 96% dioxane lignin preparation is the only acceptable one in terms of obtaining all of the lignin components. If they are not, then the 50% dioxane preparation would be acceptable. It is entirely possible that these units were released by the enzymatic digestion of the carbohydrates and are not actually a part of lignin but were just extracted with lignin. The fact that these are released from cell wall material has been confirmed by Smith and Hartley (1983).

Infrared spectra were also taken of all of the preparations in order to see whether additional information could be obtained. These spectra are shown in Figure 7 with the bands of interest being identified by their wavenumbers. The assignment of these bands is in accordance with those of Hergert (Sarkanen and Ludwig, 1971). The two bands at 1713 and 1656 cm^{-1} are due to carbonyls. The band at 1713 cm^{-1} has been assigned to carbonyl stretching in unconjugated ketone and carboxyl groups. The absorbance of this band is greatest for the dioxane-soluble lignins. The presence of acetyl groups in the 50% dioxane lignin, as already determined by NMR spectroscopy, probably accounts for the larger absorbance, observed here for this preparation over that of the 96% dioxane lignin. Most of this band, however, is probably due to ketones. Here in the infrared spectra, unlike the NMR spectra, they are grouped together in one band, thus accentuating their importance. The 1656-cm^{-1} band is also a carbonyl stretching band due to para-substituted ketones or aryl aldehydes that are only distinguishable from each other by derivatization. Here the 50% water-soluble lignin differs by having the lowest absorbance. Confirmation of the loss of *p*-coumaric acid, as suggested by the NMR spectra, is difficult to make in the infrared spectra due to the presence of may overlapping bands. The carbonyl band for the *p*-coumaric acid should appear at 1673 cm^{-1} and that for the methyl ester at 1689 cm^{-1} (Himmelsbach and Spencer, 1988). The 1595-cm^{-1} band and the next three bands are aromatic skeletal bands. Absorbances for these bands are lower for the water-soluble fractions consistent with their higher carbohydrate content and thus lower aromaticity. The ratio of the 1595-cm^{-1} band (ring stretch associated with C–O stretch) to the adjacent 1512-cm^{-1} band has been used as an indication of the syringyl to coniferyl ratio but here is complicated by the loss of PCA and the gain in carbohydrate content that offset each other. The 1265- and 1235-cm^{-1} bands have been assigned to ring breathing with C–O stretch. The 1265-cm^{-1} band has been associated with coniferyl units and the 1235-cm^{-1} band with sinapyl and *p*-coumaryl units. Here they give us no indication of a structure change among the preparations, since their absorbance ratios are near unity in all cases, only decreasing in total absorbance on going from dioxane- to water-soluble lignins. The most dramatic difference in band absorbance ratios is in that of the 1126- and 1034-cm^{-1} bands. These bands have similar high absorbance ratios for the 96% dioxane- and water-soluble lignins and lower for the 50% dioxane- and water-soluble lignins. Since *p*-coumaryl units do not show bands in this region, these bands may be more reflective of the "core" lignin structure. According to Hergert (Sarkanen and Ludwig, 1971), this would indicate that the 96% dioxane- and water-soluble lignins were more of the syringyl type and the 50% dioxane- and water-soluble lignins were more of the coniferyl type. This kind of difference is not subtantiated by any of the other absorbances observed for these components but is supported by the chemical data (Table I). Alternatively, this observation may be due to fewer units, with linear side chains being extracted into the 96% dioxane. Other than this, the infrared spectra confirm the other results that the "core" lignin structure does not change dramatically in any of the preparations. However, the two dioxane-soluble lignin preparations and the two water-soluble lignin preparations are more like each other in other respects. Except for some loss of PCA or CA and containing an acetyl group plus little more carbohydrate, the 50% dioxane lignin is representative of the lignin in the 96% dioxane lignin preparation.

It can be concluded from all of the spectral and chemical information that it would be acceptable to use 50% (v/v) dioxane–water to extract a lignin material that would be just as representative of polymeric lignin as extraction with the standard method (96% dioxane) from wheat straw and similar materials. Thus, advantage can be taken of the increased yield that this procedure provides for grasses and other herbaceous tissues that have relatively low lignin content when compared to wood. Further research is

necessary to verify this conclusion in less mature forage material and to pinpoint the major source of the phenolic acids in these materials.

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